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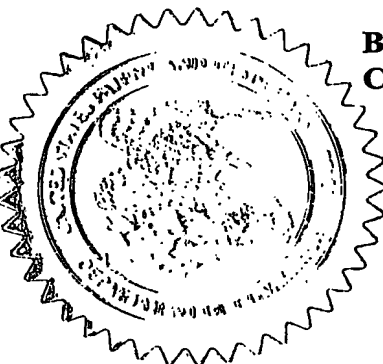
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| Given Name (first and middle [if any]) | Family Name or Surname | Residence (City and either State or Foreign Country) |
|--|------------------------|---|
| Gideon Marius | Clore | Bethesda, MD |
| Carole | Bewley-Clore | Bethesda, MD |
| John L. | Medabalimi | Bethesda, MD |

☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto**TITLE OF THE INVENTION (500 characters max)**

Novel Peptide Inhibitor of HIV Fusion That Disrupts the Internal Trimeric Coiled-Coil of gp41

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Application Data Sheet. See 37 CFR 1.76

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NIDDK, NIH, DHHS

Respectfully submitted,

SIGNATURE

Date

02/11/2003

TYPED or PRINTED NAME

Jeffrey I. Auerbach

REGISTRATION NO.

32,680

(if appropriate)

Docket Number:

03514.160

TELEPHONE

(301) 896-0600

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Title of the Invention**Novel Peptide Inhibitor of HIV Fusion That Disrupts the Internal Trimeric Coiled-Coil of gp41****5 Field of the Invention**

This invention relates to a novel peptide inhibitor of HIV fusion that disrupts the internal trimeric coiled-coil of gp41, to a pharmaceutical composition that comprise this inhibitor, and to methods of treating Immunodeficiency disease, especially HIV, that employ such a pharmaceutical composition.

10 Statement of Governmental Interest

This invention was funded by the Laboratories of Bioorganic Chemistry, Chemical Physics, NIDDK, National Institutes of Health. This work was also supported by the Intramural AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health. The United States Government has
15 certain rights to this invention.

Background of the Invention

The human immunodeficiency virus (HIV) is a pathogenic retrovirus (Varmus, H. (1988) "RETROVIRUSES," Science 240:1427-1439; Cowley S. (2001) "THE BIOLOGY OF HIV INFECTION" Lepr Rev. 72(2):212-20). It is the causative
20 agent of acquired immune deficiency syndrome (AIDS) and related disorders (Gallo, R.C. *et al.* (1983) "Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS)," Science 220(4599):865-7; Barre-Sinoussi, F. *et al.* "ISOLATION OF A T-LYMPHOTROPIC RETROVIRUS FROM A PATIENT AT RISK FOR ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)," (1983) Science
25 220:868-870; Gallo, R. *et al.* (1984) "FREQUENT DETECTION AND ISOLATION OF CYTOPATHIC RETROVIRUSES (HTLV-III) FROM PATIENTS WITH AIDS AND AT RISK FOR AIDS," Science 224:500-503; Teich, N. *et al.* (1984) "RNA TUMOR

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- VIRUSES," Weiss, R. *et al.* (eds.) Cold Spring Harbor Press (NY) pp. 949-956). HIV acts to compromise the immune system of infected individuals by targeting and infecting the CD-4⁺ T-lymphocytes that would otherwise be the major proponents of the recipient's cellular immune system response (Dagleish, A. *et al.* (1984) "THE CD4 (T4) ANTIGEN IS AN ESSENTIAL COMPONENT OF THE RECEPTOR FOR THE AIDS RETROVIRUS," *Nature* 312: 767-768, Maddon *et al.* (1986) "THE T4 GENE ENCODES THE AIDS VIRUS RECEPTOR AND IS EXPRESSED IN THE IMMUNE SYSTEM AND THE BRAIN," *Cell* 47:333-348; McDougal, J. S. *et al.* (1986) "BINDING OF HTLV-III/LAV TO T4+ T CELLS BY A COMPLEX OF THE 110K VIRAL PROTEIN AND THE T4 MOLECULE," *Science* 231:382-385). HIV infection is pandemic and HIV-associated diseases represent a major world health problem.

- Attempts to treat HIV infection have focused on the development of drugs that disrupt the viral infection and replication cycle (see, Mitsuya, H. *et al.* (1991) "TARGETED THERAPY OF HUMAN IMMUNODEFICIENCY VIRUS-RELATED DISEASE," *FASEB J.* 5:2369-2381). Such intervention could potentially inhibit the binding of HIV to cell membranes, the reverse transcription of the HIV RNA genome into DNA, the exit of the virus from the host cell and infection of new cellular targets, or inhibition of viral enzymes (see, U.S. Patent No. 6,475,491). Thus, for example, soluble CD4 has been developed in an effort to competitively block the binding of HIV to lymphocytes (Smith, D.H. *et al.* (1987) "BLOCKING OF HIV-1 INFECTIVITY BY A SOLUBLE, SECRETED FORM OF THE CD4 ANTIGEN," *Science* 238:1704-1707; Schooley, R. *et al.* (1990) "RECOMBINANT SOLUBLE CD4 THERAPY IN PATIENTS WITH THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS-RELATED COMPLEX. A PHASE I-II ESCALATING DOSAGE TRIAL," *Ann. Int. Med.* 112:247-253; Kahn, J.O. *et al.* (1990) "THE SAFETY AND PHARMACOKINETICS OF RECOMBINANT SOLUBLE CD4 (RCD4) IN SUBJECTS WITH THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS-RELATED COMPLEX. A PHASE I STUDY," *Ann. Int. Med.* 112:254-261; Yarchoan, R. *et al.* (1989) *Proc. Vth Int. Conf. on AIDS*, p564, MCP 137).

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- Likewise, drugs such as 2',3'-dideoxynucleoside analogs (e.g., azidothymidine (AZT), dideoxyinosine, dideoxycytidine, and d4thymidine have been developed to target the virus' reverse-transcriptase (Yarchan, R. *et al.* (1989) "CLINICAL PHARMACOLOGY OF 3'-AZIDO-2',3'-DIDEOXYTHYMIDINE (ZIDOVUDINE) AND RELATED DIDEOXYNUCLEOSIDES," N Engl J Med. 321(11):726-38). Recently, inhibitors of the HIV protease have been identified and used to treat HIV infection (see, U.S. Patent No. 6,472,404; Todd, S. *et al.* (2000) "HIV PROTEASE AS A TARGET FOR RETROVIRUS VECTOR-MEDIATED GENE THERAPY," Biochim Biophys Acta. 1477(1-2):168-88).
- 10 HIV infection is believed to occur through the fusion of viral-cell and cell-cell membranes. This process is mediated by the gp41 and gp120 HIV env proteins and the cellular CD4 protein. Following binding of gp120 to CD4, a conformational change occurs in the gp120/gp41 complex. This change leads to the insertion of the gp41 protein into the target membrane and ultimately to
- 15 membrane fusion. Agents that interfere with hairpin formation can inhibit HIV-1 infection (Root, M.J. *et al.* (2001) "PROTEIN DESIGN OF AN HIV-1 ENTRY INHIBITOR" *Science* 291 884-888; Kilby, J.M. *et al.* (1998) "POTENT SUPPRESSION OF HIV-1 REPLICATION IN HUMANS BY T-20, A PEPTIDE INHIBITOR OF GP41-MEDIATED VIRUS ENTRY" *Nature Medicine* 4(11) 1302-1307). Thus, various
- 20 domains of gp41 have been implicated as possible inhibitors of HIV infection (Wild, C.T. *et al.* (1994) "PEPTIDES CORRESPONDING TO A PREDICTIVE α -HELICAL DOMAIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE GP41 ARE POTENT INHIBITORS OF VIRUS INFECTION," *Proc. Natl. Acad. Sci. (U.S.A.)* 91 9770-9774; Wild, C.T. *et al.* (1992) "A SYNTHETIC PEPTIDE INHIBITOR OF HUMAN
- 25 IMMUNODEFICIENCY VIRUS REPLICATION: CORRELATION BETWEEN SOLUTION STRUCTURE AND VIRAL INHIBITION," *Proc. Natl. Acad. Sci. (U.S.A.)* 89 10537-10541).

- The gp41 region containing the naturally occurring sequence of residues 546-581 of the HIV-1 Env protein has been previously found to possess HIV-
- 30 inhibitory activity (Louis, J.M. *et al.* (2001) "DESIGN AND PROPERTIES OF NCCG-

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GP41, A CHIMERIC GP41 MOLECULE WITH NANOMOLAR HIV FUSION INHIBITORY ACTIVITY" *J. Biol. Chem.* 276 (31, Issue of August 3, 2001) 29485-29489). In addition, researchers have explored the effects of mutation in this region on membrane fusion (Weng, Y. *et al.* (1998) "MUTATIONAL ANALYSIS OF RESIDUES IN THE COILED-COIL DOMAIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 TRANSMEMBRANE PROTEIN GP41" *J. Virol.* 72(12) 9676-82).

Significantly, despite substantial research into the causes and treatment of HIV (see, for example, US20020106374a1; WO0232452a1; WO0224735a2; WO0222077a3; WO0222077a2; US6333395; US6331404; US20010047080a1; US6271198; WO0155439a1; EP0538283b2; WO0151673a2; WO0151673a1; US6258782; WO0144286a3; WO0144286a2; WO0137881a3; WO0137881a2; US6228983; EP0572737b1; US6171596; US6150088; WO0055377a1; US6093794; EP1019511a2; US6068973; WO0006599a1; US6020459; US6017536; US6013263; EP0652895b1; US5864027; US5834267; WO9820036a1; US5736391; EP0793675a1; EP0774971a1; EP0538283b1; US5576421; WO9619495a1; EP0674657a1; EP0572737a3; WO9412533a1; EP0572737a2; EP0538283a1; WO9200997a1; and WO8909785a1), suitable inhibitors of HIV fusion, that possess the ability to disrupt the internal trimeric coiled-coil of gp41 have not been identified. The present invention is directed to such inhibitors.

Summary of the Invention

The pre-hairpin intermediate of gp41 from the human immunodeficiency virus (HIV) is the target for two classes of fusion inhibitors that bind to the C-terminal region or the trimeric coiled-coil of N-terminal helices, thereby preventing formation of the fusogenic trimer of hairpins. Using rational design, two 36-residue peptides, N36^{Mut(e,g)} and N36^{Mut(a,d)}, were derived from the parent N36 peptide comprising the N-terminal helix of the gp41 ectodomain (residues 546-581 of HIV-1 envelope), characterized by analytical ultracentrifugation and CD, and assessed for their ability to inhibit HIV fusion using a quantitative vaccinia virus-based fusion assay. N36^{Mut(e,g)} contains nine amino acid substitutions designed to disrupt

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interactions with the C-terminal region of gp41 while preserving contacts governing the formation of the trimeric coiled-coil. N36^{Mut(a,d)} contains nine substitutions designed to block formation of the trimeric coiled-coil but retains residues that interact with the C-terminal region of gp41. N36^{Mut(a,d)} is monomeric, is largely random coil, does not interact with the C34 peptide derived from the C-terminal region of gp41 (residues 628-661), and does not inhibit fusion. The trimeric coiled-coil structure is therefore a prerequisite for interaction with the C-terminal region of gp41. N36^{Mut(c,g)} forms a monodisperse, helical trimer in solution, does not interact with C34, and yet inhibits fusion about 50-fold more effectively than the parent N36 peptide (IC₅₀ ~ 308 nM *versus* ~16 μM). These results indicate that N36^{Mut(c,g)} acts by disrupting the homotrimeric coiled-coil of N-terminal helices in the pre-hairpin intermediate to form heterotrimers. Thus N36^{Mut(c,g)} represents a novel third class of gp41-targeted HIV fusion inhibitor. A quantitative model describing the interaction of N36^{Mut(c,g)} with the pre-hairpin intermediate is presented.

This invention thus relates to a novel peptide inhibitor of HIV fusion that disrupts the internal trimeric coiled-coil of gp41, to a pharmaceutical composition that comprise this inhibitor, and to methods of treating Immunodeficiency disease, especially HIV, that employ such a pharmaceutical composition.

The invention is more fully described in Bewley, C.A., Louis, J.M., Ghirlando, R. and Clore, G.M. (2002) "DESIGN OF A NOVEL PEPTIDE INHIBITOR OF HIV FUSION THAT DISRUPTS THE INTERNAL TRIMERIC COILED-COIL OF GP41," *J. Biol. Chem.* 277 (16, Issue of April 19, 2002) 14238-14245), herein incorporated by reference in its entirety. An on-line version of this publication was published on February 21, 2002 (M201453200); see also, Sanders, R.W. *et al.* (July 29, 2002) "STABILIZATION OF THE SOLUBLE, CLEAVED, TRIMERIC FORM OF THE ENVELOPE GLYCOPROTEIN COMPLEX OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1," *J. Virol.* 76:8875-8889; Follis, K.E. *et al.* (June 14, 2002) "GENETIC EVIDENCE THAT INTERHELICAL PACKING INTERACTIONS IN THE GP41 CORE ARE CRITICAL FOR

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TRANSITION OF THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE GLYCOPROTEIN TO THE FUSION-ACTIVE STATE," J. Virol. 76:7356-7362.

In detail, the invention concerns polypeptides that increase the stability of the peptide-gp41 heterotrimer over the peptide and gp41 homotrimers. The invention particularly concerns the N36^{Mut(e,g)} peptide, having the amino acid sequence:

SGIDQEQNNL TRLIEAQIHE LQLTQWKIKQ LLARIL (SEQ ID NO:1)
1 10 20 30 36

as well as mutated forms of this sequence that yield polypeptides that increase the stability of the peptide-gp41 heterotrimer over the peptide and gp41 homotrimers. Particularly preferred mutated forms of this sequence are those that possess single, double, etc. conservative point mutations at positions a or d of the helical wheel representation of this sequence (see Figure 2a). Methods of making such mutated forms of an amino acid sequence are well known in the art.

The invention further concerns the use of such polypeptides in the treatment of HIV transmission, and in the treatment of AIDS. The invention additionally concerns a method of attenuating the transmission or infection of HIV into cells, and of treating AIDS, comprising providing one or more of such peptide(s) to such cells.

Brief Description of the Figures

Figures 1a-1b show a schematic model illustrating the site of action of different HIV fusion inhibitors that target the ectodomain of gp41. *a*, the fusogenic state of gp41 (*bottom*) consists of a trimer of hairpins comprising an internal trimeric, helical coiled-coil of the N-region surrounded by helices derived from the C-region (4-8). The inhibitors target a pre-hairpin intermediate state (*top*) in which the N- and C- regions of gp41 are not yet associated (2). In the pre-hairpin intermediate state, the N-region is thought to consist of a trimeric, parallel helical coiled-coil; the fusion peptide (*green*) located at the N terminus of the ectodomain of gp41 is inserted into the target cell membrane; the C-region of gp41 is anchored

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to the viral membrane by a transmembrane segment (*purple*). Peptides derived from the C-region, such as C34 (11), bind to the N-region in its trimeric coiled-coil state; the proteins N_{CCG}-gp41 (19) and 5-helix (20), which expose either the complete or a portion of the N-region trimeric coil-coil in a stable form, bind to the C-region. N36^{Mut(e,g)}, the subject of the present article, has been designed to remove the interaction surface between the N- and C-regions and therefore can only interact with the N-region in a monomeric form, thereby disrupting the homotrimeric coiled-coil N-region and resulting in the formation of heterotrimers. In all three instances, the fusion inhibitors block the formation of the trimer of hairpins, thereby preventing apposition of the viral and target cell membranes. *b*, as a consequence of the existence of a monomer-trimer equilibrium for the trimeric coiled-coil of N-helices, the interaction of homotrimeric N36^{Mut(e,g)} (*yellow*) with the fusion-competent homotrimeric pre-hairpin intermediate (N-helices) results in subunit exchange and the formation of fusion-incompetent heterotrimers.

Figures 2a-2b show the design of a peptide that disrupts the internal N-region trimeric coiled-coil in the pre-hairpin intermediate state of gp41. *a*, helical wheel representation illustrating the interaction between the N- and C-regions of gp41 in the trimer of hairpins as observed in the solution (4) and crystal (5-8) structures of the fusogenic/postfusogenic state of the ectodomain of gp41. The intermolecular contacts between the N-helices occur between *positions a* and *d* of the helical wheel. Contacts between the N- and C-helices (intra- and intermolecular) involves residues at *positions e* and *g* of the N-helices and *positions a* and *d* of the C-helices. *b*, peptide sequences. The N36 peptide comprises residues 546-581 of the N-region of HIV-1 gp41, and the C34 peptide comprises residues 628-661 of the C-region of HIV-1 gp41. N36 and C34 associate to form a six-helix bundle whose structure has been solved crystallographically (5). In the N36^{Mut(e,g)} mutant, the residues at *positions e* and *g* of N36 have been substituted by residues at *positions e* and *g*, respectively, of C34; this effectively removes the interaction surface with C34 but preserves the contacts necessary to form a trimeric coiled-coil of N-helices. In the N36^{Mut(a,d)} mutant, the residues at *positions a* and *d* of N36 have been substituted by residues at *positions f* and *c*,

respectively, of C34; this removes the contacts necessary to form the trimeric coil-coil of N-helices but preserves the interaction sites with C34.

Figures 3a-3b show the characterization of $N36^{Mut(e,g)}$ and $N36^{Mut(a,d)}$. *a*, results of analytical ultracentrifugation on $N36^{Mut(e,g)}$ (*a*, *top*) and $N36^{Mut(a,d)}$ (*a*, *lower*). Sedimentation equilibrium profiles, plotted in terms of $\ln(A_{280})$ versus the square of the radius (r^2) (*bottom panel*), are shown; also shown in the *top two panels* is the distribution of residuals between calculated and experimental data for best fits to a monomer of $N36^{Mut(a,d)}$ and a trimer of $N36^{Mut(e,g)}$. The concentrations (in monomer) of $N36^{Mut(a,d)}$ and $N36^{Mut(e,g)}$ are $\sim 140 \mu M$ ($A_{280} \sim 0.8$) and $124 \mu M$ ($A_{280} \sim 0.7$), respectively. The calculated molecular masses are 3660 ± 80 Da for $N36^{Mut(a,d)}$, which corresponds to a monomer, and $12,040 \pm 200$ Da for $N36^{Mut(e,g)}$, which corresponds to a trimer. An independent run for $N36^{Mut(e,g)}$ at a concentration of $36 \mu M$ in monomer ($A_{280} \sim 0.2$) yielded a molecular mass of $12,500 \pm 180$ Da. *b*, CD spectrum of $N36^{Mut(e,g)}$ and $N36^{Mut(a,d)}$. The calculated helical content is $\sim 80\%$ for $N36^{Mut(e,g)}$ and $\sim 20\%$ for $N36^{Mut(a,d)}$. *deg*, degrees.

Figure 4 shows the inhibition of HIV Env-mediated cell fusion by $N36^{Mut(e,g)}$, $N36^{Mut(a,d)}$, and N36. *circles*, $N36^{Mut(e,g)}$; *solid squares*, $N36^{Mut(a,d)}$; *black open circles*, N36. The *solid lines* represent best fits to the data using the simple activity relationship: $\%fusion = 100/(1+[I]/IC_{50})$ where $[I]$ is the inhibitor concentration. The IC_{50} values for $N36^{Mut(e,g)}$ and N36 are 308 ± 22 nM and $16 \pm 2 \mu M$, respectively. $N36^{Mut(a,d)}$ displays no inhibitory activity at the concentrations tested.

Figures 5a-5e model the inhibition of HIV Env-mediated cell fusion by $N36^{Mut(e,g)}$. *a*, mechanistic scheme. L, LL, and LLL are the monomeric, homodimeric, and homotrimeric forms, respectively, of the ligand $N36^{Mut(e,g)}$; M, MM, and MMM are the monomeric, homodimeric, and homotrimeric forms, respectively, of the prefusion intermediate of gp41 bound on the surface of the cell; ML is the heterodimeric species formed by the interaction of M and L; MML and MLL are the heterotrimeric species. $K_{ref}^{L-trimer}$ is the experimentally measured

equilibrium association constant for the monomer-trimer equilibrium of the ectodomain of gp41 in free solution ($4.8 \times 10^{11} \text{ M}^{-2}$; Ref. 21) given by the product of K_1 and K_2 with $K_2 \gg K_1$ (since trimer formation is highly cooperative, and only monomer and trimer species can be detected by analytical ultracentrifugation). (K_1 was arbitrarily set to 10^4 M^{-1} , yielding a value of $4.8 \times 10^7 \text{ M}^{-1}$ for K_2 .) The factors α , β , and λ relate the equilibrium association constants for

homotrimerization of L ($K_{\text{homo,L}}^{\text{trimer}}$), heterotrimerization of M and L ($K_{\text{hetero,ML}}^{\text{trimer}}$), and homodimerization of M ($K_{\text{homo,M}}^{\text{trimer,app}}$) to $K_{\text{ref}}^{\text{trimer}}$. The factor λ serves to convert the concentrations of species in the membrane to their bulk solution concentrations and, in addition, subsumes any energetic differences between trimerization of the pre-hairpin intermediate of gp41 in the membrane and trimerization of the ectodomain of gp41 measured in free solution. The various numerical factors in front of the equilibrium constants are statistical factors related to symmetry considerations involved in the formation of homo- and hetero-oligomeric species.

b , variation in the optimized values of α and β derived by nonlinear least-squares optimization as a function of λM_T , where M_T is the total concentration of protein (monomer units) in bulk solution. The *vertical bars* represent the error in the fitted parameters. c , comparison of the experimental fusion data (*solid circles*) with the best fit theoretical curves calculated for $\lambda M_T = 1.5 \times 10^{-5} \text{ M}$ (*solid line*) and $1.5 \times 10^{-4} \text{ M}$ (*dashed line*). (For a value of 10 pM for M_T , the corresponding values of λ are 1.5×10^6 - 1.5×10^7 , respectively). The percentage of fusion activity is given by $100[\text{MMM}]_{L_T}/[\text{MMM}]_{L_T=0}$. Note that the two theoretical curves are essentially indistinguishable not only from each other but also from a simple Langmuir isotherm. Calculated fractional concentrations of various species as a function of total protein concentration, M_T (d), and total ligand concentration, L_T (e). The fraction of dimeric species (*i.e.* $2[\text{MM}]/M_T$, $[\text{ML}]/M_T$, and $2[\text{LL}]/L_T$) is less than 1% for all values of L_T . The curves obtained for $\lambda M_T = 1.5 \times 10^{-5} \text{ M}$ and $1.5 \times 10^{-4} \text{ M}$ are shown as *solid* and *dashed lines*, respectively.

Description of the Preferred Embodiments

The abbreviations used herein are: Env, viral envelope glycoprotein(s); HIV, human immunodeficiency virus; gp120, surface envelope glycoprotein of HIV; gp41, transmembrane subunit of HIV envelope; N36 and C34, peptides
 5 encompassing residues 546-581 and 628-661 of HIV-1 Env, respectively; N36^{Mut(e, g)}, peptide derived from N36 that contains nine substitutions at positions *e* and *g* of the helical wheel (defined in the context of the gp41 trimer of hairpins structure) corresponding to residues 549, 551, 556, 558, 563, 565, 570, 572, and 577 of HIV-1 Env; N36^{Mut(a, d)}, peptide derived from N36 that contains nine
 10 substitutions at positions *a* and *d* of the helical wheel (defined in the context of the gp41 trimer of hairpins structure) corresponding to residues 552, 555, 559, 562, 566, 569, 573, 576, and 580 of HIV-1 Env.

Virus-cell and cell-cell fusion mediated by the viral envelope glycoproteins (Env)¹ (1) gp41 and gp120 constitute the first step of infection and dissemination,
 15 respectively, of the human immunodeficiency virus (HIV) and hence represent a promising target for the development of antiviral therapeutics (2). Following binding of gp120 to CD4 and a chemokine receptor, a conformational change occurs in the gp120/gp41 oligomer that leads to insertion of the fusion peptide of gp41 into the target membrane and ultimately membrane fusion (2, 3). The
 20 structure of the ectodomain of both HIV and simian immunodeficiency virus gp41 in its fusogenic/postfusogenic state has been solved by NMR (4) and crystallography (5-8) and shown to consist of a trimer of hairpins. Each subunit comprises long N- and C-terminal helices connected by a 25-30-residue loop. The N-helices form a parallel, trimeric coiled-coil in the interior of the complex
 25 surrounded by the C-terminal helices oriented antiparallel to the N-terminal helices (Fig. 1a, bottom). Peptides derived from the C- and N-helices inhibit Env-mediated fusion at nanomolar and micromolar concentrations, respectively (9-12). These peptides do not bind the fusion-active or postfusogenic state of gp41 as represented by the ectodomain of gp41 free in solution and are thought to interact with a pre-hairpin intermediate (2, 13, 14) in which the N- and C-helices are not associated
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and the trimeric coiled-coil of N-helices is exposed (Fig. 1a, top left). Peptides derived from the C-terminal helix, such as C34 (residues 628-661 of HIV-1 Env) and T20 (residues 638-673 of HIV-1 Env; currently in phase III clinical trials (15, 16)) target the exposed face of the trimeric coiled-coil of N-helices (11, 13, 14, 17, 18). Engineered constructs such as the chimeric protein Nccg-gp41 (19), which features an exposed, stable, disulfide-linked, trimeric coiled-coil of N-helices grafted onto the minimal, thermostable ectodomain of gp41; peptides in which the trimeric coiled-coil of N-helices is stabilized by fusion to the GCN4 trimeric coiled-coil (12); and the protein 5-helix (20), in which the internal trimeric coiled-coil of N-helices is surrounded by only two C-helices, specifically target the C-region in the pre-hairpin intermediate state (Fig. 1a, top left). In both instances, packing of the C-region onto the trimeric coiled-coil of N-helices is blocked, and hairpin formation is inhibited. Although the ectodomain of gp41 in free solution is highly thermostable (with a T_m in excess of 100 °C) (21), it has been shown to exist as a monomer-trimer equilibrium (21, 22). In the context of the fusion process, the trimeric coiled-coil of N-helices in the pre-hairpin intermediate state may also exist as a monomer-trimer equilibrium (4, 22, 23). If this is indeed the case, blocking the formation of the fusion-competent, homotrimeric coiled-coil of N-helices may provide another molecular target for inhibiting HIV cell fusion. In this article, we present the design and characterization of a peptide, derived from the N-helix of gp41, in which the sites of interaction with the C-helices have been mutated, but the sites of intermolecular contacts between the N-helices have been preserved. This peptide, which we term N36^{Mut(c,g)}, is about 50-fold more effective in inhibiting HIV Env-mediated cell fusion than the N36 peptide (residues 546-581 of HIV-1 Env) of gp41 from which it was derived. These data strongly suggest that the homotrimeric coiled-coil of N-helices in the pre-hairpin state can be disrupted.

The present invention additionally provides a method of treating immunodeficiency virus infection which comprises administering an effective amount of such inhibitor peptides of the present invention to a mammal either infected with such virus or at risk of such infection. Thus, the invention provides a treatment for: humans either infected with HIV or at risk of such infection; felines

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either infected with feline immunodeficiency virus or at risk of such infection; and simians either infected with simian immunodeficiency virus or at risk of such infection.

The effective amount of the inhibitor peptides of the present invention to be administered to the mammal, such as a human, is a subtoxic amount. Preferably, the subtoxic amount is an amount that produces little or no killing of uninfected cells. More preferably, the subtoxic amount is an amount that produces little or no effect on the morphology of uninfected cells. The inhibitor peptides of the present invention can be administered in a single dose or in multiple doses in a given period of time (e.g., a single daily dose or two or more doses a day). The subtoxic dose depends on the age, weight, general health, and extent of infection being treated. The inhibitor peptides of the present invention may be administered alone, or in combination with other immunodeficiency virus treatment regimens.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. The administration of such compounds may be for either a "prophylactic" or "therapeutic" purpose. The compositions of the present invention are said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant to provide a therapy for an actual infection. When provided therapeutically, the compound is preferably provided at (or shortly after) the onset of a symptom of actual infection. The therapeutic administration of the compound serves to attenuate any actual infection. The compositions of the present invention are said to be administered in a "prophylactically effective amount" if the amount administered is physiologically significant to provide a therapy for an potential infection. When provided prophylactically, the compound is preferably provided in advance of any immunodeficiency virus infection or symptom thereof. The prophylactic administration of the compound serves to prevent or attenuate any subsequent infection.

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The compounds of the present invention can be administered in conventional solid or liquid pharmaceutical administration forms, for example, as uncoated or (film-) coated tablets, capsules, powders, granules, suppositories or solutions. The active substances can, for this purpose, be processed with conventional pharmaceutical aids such as tablet binders, fillers, preservatives, tablet disintegrants, flow regulators, plasticizers, wetting agents, dispersants, emulsifiers, solvents, sustained release compositions, antioxidants and/or propellant gases. The therapeutic compositions obtained in this way typically contain from about 0.1% to about 90% by weight of the active substance.

The inhibitor peptides of the present invention may be provided to recipients alone or in combination with one or more other pharmaceutical compositions (such as those that inhibit the binding of HIV to cell membranes, those that inhibit HIV reverse transcriptase, those that inhibit the exit of the virus from the host cell and/or the infection of new cellular targets, or those that inhibit viral enzymes (e.g., drugs such as 2',3'-dideoxynucleoside analogs (e.g., azidothymidine (AZT), dideoxyinosine, dideoxycytidine, and d4thymidine, etc.)).

Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Example 1 Experimental Methods

Peptides-- All peptides (Fig. 2b), purchased from Commonwealth Biotechnologies (Richmond, VA), were synthesized on a solid phase support, purified by reverse phase high pressure liquid chromatography, and verified for purity by mass spectrometry and amino acid composition. All peptides bear an acetyl group at the N terminus and an amide group at the C terminus. Concentrations of peptides were determined spectrophotometrically: the calculated A_{280} values (1-cm path length) for a concentration of 1 mg/ml N36, N36^{Mut(c,g)},

N36^{Mut(a,d)}, and C34 are 1.35, 1.31, 1.34, and 2.90, respectively. The corresponding molecular masses are 4160, 4293, 4182, and 4286 Da, respectively.

Circular Dichroism-- CD spectra of peptides (at a concentration corresponding to 0.7-0.8 A_{280}) were recorded at 25 °C on a JASCO J-720 spectropolarimeter using a 0.05-cm path length cell. Quantitative evaluation of secondary structure from the CD spectrum was carried out using the program CDNN (www.bioinformatik.biochemtech.uni-halle.de/cd_spect/index.html; Ref. 24).

Sedimentation Equilibrium-- Sedimentation equilibrium experiments were conducted at 20.0 °C and three different rotor speeds (16,000, 20,000, and 24,000) on a Beckman Optima XL-A analytical ultracentrifuge. Peptide samples were prepared in 50 mM sodium formate buffer (pH = 3) and loaded into the ultracentrifuge cells at nominal loading concentrations of ~0.2 and 0.7-0.8 A_{280} . Data were analyzed in terms of a single ideal solute to obtain the buoyant molecular mass, $M(1 - \nu\rho)$, using the Optima XL-A data analysis software (Beckman). The value for the experimental molecular mass M was determined using calculated values for the density ρ (determined at 20 °C using standard tables) and partial specific volume ν (calculated on the basis of amino acid composition (25)).

Cell Fusion Assay-- Inhibition of HIV Env-mediated cell fusion by peptides was carried out as described previously (19) using a modification (26) of the vaccinia virus-based reporter gene assay (using soluble CD4 at a final concentration of 200 nM). B-SC-1 cells were used for both target and effector cell populations. Target cells were co-infected with vCB21R-LacZ and vCBYF1-fusin (CXCR4), and effector cells were co-infected with vCB41 (Env) and vP11T7gene1 at a multiplicity of infection of 10. For inhibition studies, peptides were added to an appropriate volume of Dulbecco's modified Eagle's medium (2.5%) and phosphate-buffered saline to yield identical buffer compositions (100 μ l) followed by addition of 1×10^5 effector cells (in 50 μ l of medium) per well. After incubation for 15 min,

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1 $\times 10^5$ target cells (in 50 μ l) and soluble CD4 were added to each well. Following
 2.5 h of incubation, β -galactosidase activity of cel⁺ lysates was measured (A_{570} ;
 Molecular Devices 96-well spectrophotometer) upon addition of chlorophenol red-
 β D-galactopyranoside (Roche Molecular Biochemicals). The curves for %fusion
 5 versus peptide inhibitor concentration were fit by nonlinear least-squares
 optimization using the program FACSIMILE (27, 28).

Example 2 Design of Peptide Inhibitors

The helical wheel diagram in Fig. 2a illustrates the interactions between the
 10 N-helices and between the N- and C-helices as observed in both the NMR (4) and
 x-ray (5-8) structures of the fusogenic/postfusogenic state of the ectodomain of
 gp41. Internal contacts between the N-helices involve positions *a* and *d* of the
 helical wheel (5). Each C-helix interacts with two N-helices (one intra- and the
 other intersubunit): these contacts involve positions *e* and *g* of the N-helices and
 15 positions *a* and *d* of the C-helix (5). The first crystal structure of the HIV-1 gp41
 ectodomain core consisted of a complex of N36 and C34 peptides comprising
 residues 546-581 and 628-661, respectively, of HIV-1 Env (5). Using the N36 and
 C34 peptides as starting points, we designed two peptides: N36^{Mut(e,g)}, which can
 only undergo self-association but cannot interact with C34, and N36^{Mut(a,d)}, which
 20 can no longer self-associate but could potentially still interact with C34 (Fig. 2b).
 In the case of N36^{Mut(e,g)}, the residues at positions *e* and *g* of N36 have been
 replaced by residues at positions *e* and *g* of C34. Since the latter residues are
 located on the external surface of C34 in the context of the ectodomain gp41 core
 (4-8) and since C34 on its own is monomeric (29), this set of substitutions will
 25 prevent any interaction between N36^{Mut(e,g)} and the C-region of gp41 in its pre-
 hairpin intermediate state while preserving the intermolecular contacts required to
 form the trimeric coiled-coil of N-helices. In the case of N36^{Mut(a,d)}, the residues at
 positions *a* and *d* of N36 have been substituted by residues at positions *f* and *c* of
 C34, which are located on the solvent-exposed face of the ectodomain core of gp41
 30 (4-8), thereby removing the intermolecular contacts required to form the trimeric
 coiled-coil of N-helices.

Example 3
Biophysical Characterization of N36^{Mut(e,g)} and N36^{Mut(a,d)}

The results of analytical ultracentrifugation on N36^{Mut(e,g)} and N36^{Mut(a,d)} are presented in Fig. 3a. N36^{Mut(e,g)} behaves as a single monodisperse species at concentrations of ~36 μ M (in monomer; $A_{280} \sim 0.2$) and ~124 μ M (in monomer; $A_{280} \sim 0.7$) with a molecular mass of ~12,000-12,500 Da, corresponding to a trimer. In this context it is worth noting that N36 on its own aggregates and does not form a well defined trimer (12),² presumably due to further self-association involving the predominantly hydrophobic residues at positions e and g, which have been substituted by predominantly hydrophilic residues in N36^{Mut(e,g)} (Fig. 2b). N36^{Mut(a,d)} also behaves as a single monodisperse species at a concentration of ~140 μ M ($A_{280} \sim 0.8$), but its molecular mass is only ~3700 Da, corresponding to a monomer.

CD spectra of N36^{Mut(e,g)} and N36^{Mut(a,d)} are shown in Fig. 3b. N36^{Mut(e,g)} displays a double minimum at 208 and 222 nm, characteristic of an α -helix and quantification of the CD data (24) indicates a helical content of ~80%. N36^{Mut(a,d)}, on the other hand, is largely random coil (characterized by a minimum around 200 nm) with a small amount of α -helix (~20%).>

No evidence of interaction between either N36^{Mut(e,g)} or N36^{Mut(a,d)} and C34 was detected by either analytical ultracentrifugation or CD. The absence of interaction between N36^{Mut(e,g)} and C34 is exactly as predicted from the design since the points of contact with C34 have been mutated (cf. Fig. 2). The absence of interaction between N36^{Mut(a,d)} and C34 was initially somewhat surprising since the residues that contact C34 in the context of the fusogenic/postfusogenic state of the gp41 ectodomain were preserved. This result therefore indicates that C34 can only form a complex with a stable trimeric coiled-coil of N-helices. From a structural standpoint, this is readily understood since each C-helix contacts two N-helices of the trimeric coiled-coil (one intramolecular and the other intersubunit; cf. Fig. 2a), and the buried surface area for each of the two interactions is comparable.

To exclude the remote possibility that N36^{Mut(e,g)} could behave in a manner analogous to C34 and bind to the surface of the trimeric coiled-coil of N-helices in the pre-hairpin intermediate of gp41, the interaction of N36^{Mut(e,g)} with the engineered protein NCCG-gp41 was examined. NCCG-gp41 is a chimeric protein that features an exposed trimeric coiled-coil of N-helices that is stabilized both by fusion to a minimal thermostable ectodomain of gp41 and by engineered intersubunit disulfide bonds (19). The exposed trimeric coiled-coil of N-helices in NCCG-gp41 mimics that of the pre-hairpin intermediate of gp41, but in contrast to native gp41, the N-helices cannot dissociate since they are covalently tethered by disulfide bonds. Analytical ultracentrifugation on various mixtures of N36^{Mut(e,g)} and NCCG-gp41 in ratios of 4.5:1 and 11.7:1 (24 μ M N36^{Mut(e,g)} plus 5.3 μ M NCCG-gp41 and 51 μ M N36^{Mut(e,g)} plus 4.4 μ M NCCG-gp41, respectively, with concentrations expressed in trimer) provided no evidence of any interactions between these two molecules, and the data were readily accounted for by a mixture of two ideal species.

Inhibition of HIV Env-mediated Cell Fusion-- The results of a quantitative vaccinia virus-based reporter gene assay (26) for HIV Env-mediated cell fusion are shown in Fig. 4. N36 inhibits fusion with an IC₅₀ of 16 ± 2 μ M in agreement with previous results (19). N36^{Mut(e,g)} inhibits fusion with an IC₅₀ 308 ± 22 nM. Thus N36^{Mut(e,g)} is ~50-fold more active in inhibiting fusion than N36. N36^{Mut(a,d)}, on the other hand, fails to inhibit fusion even at concentrations as high as 0.1 mM. The lack of any fusion-inhibitory activity for N36^{Mut(a,d)} is exactly as predicted from the biophysical data since N36^{Mut(a,d)} does not self-associate and does not interact with C34.

Since N36^{Mut(e,g)} forms a well defined trimeric species that does not interact with either C34 or the chimeric protein NCCG-gp41 (in which the N-helices of the solvent-exposed trimeric coil-coil are covalently linked by interhelical disulfide bonds), it must target the N-region of the pre-hairpin intermediate by forming fusion-incompetent heterotrimers (Fig. 1b). Analytical ultracentrifugation on the ectodomain of gp41 indicates the presence of only monomer and trimer species in

equilibrium (21, 22); that is, assembly of the trimer is a highly cooperative process. The fusion-inhibitory activity of N36^{Mut(e,g)} therefore indicates the presence of a dynamic equilibrium between monomeric and trimeric forms of membrane-bound gp41 that allows subunit exchange to take place in the pre-hairpin intermediate state. The rate of exchange between these species must be sufficiently fast to permit efficient heterotrimer formation within the lifetime (~15 min) of the pre-hairpin intermediate (13, 30, 31).

Example 4 Modeling Inhibition of HIV Env-mediated Cell Fusion by N36^{Mut(e,g)}

10 The inhibition curve for N36^{Mut(e,g)} is well fit by a simple Langmuir isotherm given by %fusion = 100/(1 + [N36^{Mut(e,g)}]/IC₅₀) (Fig. 4). Yet, mechanistically, the interaction of N36^{Mut(e,g)} with the pre-hairpin intermediate of gp41 is far more complex, involving multiple species in different homo- and hetero-oligomerization states. The simplest scheme describing the situation is
15 presented in Fig. 5a. L and M represent N36^{Mut(e,g)} and the pre-hairpin intermediate of gp41 in their monomeric forms, respectively; LL and MM are homodimers; ML is a heterodimer; LLL and MMM are homotrimers; and MML and MLL are heterodimers. We assume that only the homotrimer MMM is fusion-active, and the fraction fusion activity is given by the ratio of [MMM]_{LT}/[MMM]_{LT=0}. The
20 interactions between ligand (in its various oligomerization states) and membrane-bound protein (in its various homo- and hetero-oligomeric states) are described by their respective bulk solution concentrations. The interactions involving only membrane-bound species, however, are dependent on their concentrations in the two-dimensional membrane (*i.e.* number of molecules per unit area) that are much
25 higher than their concentrations in bulk solvent. In terms of thermodynamics, all equilibria in Fig. 5a can be related to the species concentrations in bulk solvent by multiplying the relevant equilibrium constants by a factor λ to yield appropriate apparent equilibrium constants (Fig. 5a, middle panel).

30 The measured equilibrium association constant K_{ref}^{Lrimer} for the ectodomain of HIV-1 gp41 in free solution (*i.e.* the trimer of hairpins) is $4.8 \times 10^{11} \text{ M}^{-2}$ and is

given by the product of the equilibrium association constants K_1 (monomer-dimer equilibrium) and K_2 (dimer-trimer equilibrium) (Fig. 5a, bottom panel). Since trimerization of the gp41 ectodomain is highly cooperative (21, 22), $K_2 \gg K_1$. Taking $K_{\text{ref}}^{\text{trimer}}$ as a reference point, the overall equilibrium association constant

5 between monomeric and homotrimeric species of L is given by $\alpha K_{\text{ref}}^{\text{trimer}}$, between monomeric and homotrimeric species of M by $\lambda K_{\text{ref}}^{\text{trimer}}$ (note that λ also subsumes any difference in the energetics of trimerization between the pre-hairpin intermediate in the membrane and the ectodomain of gp41 in free solution), and between monomeric species of L and M and heterotrimeric species of M and L by

10 $3\beta K_{\text{ref}}^{\text{trimer}}$ (where the factor 3 is a statistical factor). The scheme in Fig. 5a has three unknowns: α , β , and λM_T , where M_T is the total protein concentration (in monomer units). The data, however, are insufficient to determine all three parameters independently. Nonlinear least-squares fitting to the experimental data, optimizing the values of α and β , was carried out for values of λM_T ranging from 1.5×10^{-7} to

15 1.5×10^{-3} M (which corresponds to values of λ of 1.5×10^4 – 1.5×10^8 for $M_T = 10$ pM, the probable concentration of protein in bulk solution, estimated on the basis of a concentration of 5×10^3 cells/ μl and ~ 5000 gp41 trimers/cell). (Note that the concentrations of the various species in the scheme shown in Fig. 5a as a function of total ligand concentration, L_T , were calculated numerically by

20 integration of the differential equations describing the reactions to essentially infinite time.) The optimized values of α and β depend on the product λM_T , and the results are therefore equally valid for a wide range of M_T concentrations. The data can be equally well fitted for values of λM_T ranging from 10^{-7} to 10^{-3} M with α varying from ~ 10 to 0.1 and β varying from 1 to 10 (Fig. 5b). Best fits to the

25 experimental fusion inhibition data for $\lambda M_T = 1.5 \times 10^{-5}$ and 1.5×10^{-4} M are shown in Fig. 5c; the optimized values of α are 1.07 and 0.34 (with error estimates of $\sim 40\%$), respectively, and of β are 2.97 and 5.76 (with error estimates of 10%), respectively. The resulting curves are essentially indistinguishable from each other as well as from that obtained with a Langmuir isotherm. The occupancy of the

30 various species relative to M_T and L_T are shown in Fig. 5, d and e, respectively. For this set of parameters, the fraction M in the trimeric state in the absence of ligand is

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~86% for $\lambda M_T = 1.5 \times 10^{-5}$ M and ~97% for $\lambda M_T = 1.5 \times 10^{-4}$ M; the value of L_T at which 50% of L is monomeric is $\sim 2 \times 10^{-6}$ and 5×10^{-6} M, respectively. The occupancy of homodimeric ligand is less than 1% of L_T ; likewise the occupancy of homodimeric (MM) and heterodimeric (LM) protein is less than 1% of M_T for all values of L_T . Both MML and MLL heterotrimers are formed with the MML heterotrimer peaking at concentrations of L_T slightly less than that at which 50% of the ligand is monomeric.

The above calculations reveal two important findings. First, despite the complexities introduced by multiple homo- and hetero-oligomerization states, which might lead one to predict a complex relationship between fusion and total ligand concentration, a scheme such as that depicted in Fig. 5a can still yield rather simple inhibition data that is readily characterized by a Langmuir isotherm. Second, the values for the various equilibrium constants for trimerization required to best fit the experimental fusion data are entirely compatible with the experimentally measured value for the equilibrium constant for trimerization of the ectodomain of HIV-1 gp41 in solution.

In the best fit calculations described above and depicted in Fig. 5, only the homotrimeric form of the pre-hairpin intermediate of gp41, MMM, is considered to be fusion-active. If the calculations are repeated assuming that the heterotrimer, MML, containing only one molecule of N36^{Mut(e,g)}, is also fusion-active, the resulting theoretical curves do not reproduce the experimental data. One can therefore conclude that the energetics of formation of a five-helix bundle comprising a heterotrimeric internal coiled-coil consisting of two N-helices of gp41 and one N36^{Mut(e,g)} helix surrounded by two C-helices of gp41 is not sufficiently favorable to bring the target and viral membranes into sufficiently close proximity for fusion to take place.

Example 5
Engineering Of Peptides Derived From The N-Helix Of The Ectodomain Of gp41

Using rational design, two peptides have been engineered derived from the N-helix of the ectodomain of gp41. The parent peptide, N36, corresponds to residues 546-581 of HIV-1 Env and encompasses the N-terminal helix of gp41. The N36^{Mut(a,d)} peptide was designed to remove interactions leading to self-association and the formation of a trimeric coiled-coil of N-helices while preserving those residues that interact with the C-helix of the ectodomain of gp41.

10 The absence of any fusion-inhibitory activity of N36^{Mut(a,d)} leads us to conclude that the C-region of gp41 can only interact with a trimeric coiled-coil of N-helices. The N36^{Mut(e,g)} peptide was designed to preserve the interactions leading to self-association while replacing those residues that interact with the C-region. N36^{Mut(e,g)} forms a monodisperse trimer in solution that does not interact with the

15 C-region of gp41 and yet still inhibits fusion about 50-fold more effectively than the native gp41 sequence (*i.e.* N36) from which it was derived. These results can only be explained by the existence of a dynamic equilibrium between monomeric and trimeric coiled-coil forms of the N-region of gp41 in the pre-hairpin intermediate on a time scale sufficiently fast to permit subunit exchange and the

20 consequent formation of heterotrimers of the N-helices of gp41 and N36^{Mut(e,g)}. Thus, N36^{Mut(e,g)} disrupts the homotrimeric coiled-coil of N-helices in the pre-hairpin intermediate state of gp41 and represents a novel third class of gp41-targeted fusion inhibitor. The other two classes of inhibitors bind to either the homotrimeric coiled-coil of N-helices (*e.g.* C34 and T20) or to the exposed C-

25 region (*e.g.* NCCG-gp41 and 5-helix) of gp41 in the pre-hairpin intermediate state. Since C34 (and presumably T20) also binds to NCCG-gp41 and 5-helix (19, 20), these two classes of inhibitors antagonize each other. In contrast, one would predict that the N36^{Mut(e,g)} class of inhibitors should act either additively or synergistically with either of the other two classes. Therefore, N36^{Mut(e,g)} may represent a

30 promising lead for the design of clinically effective, novel fusion inhibitors.

All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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R. (1998) J. Cell Biol. 140, 315-323

15 While the invention has been described in connection with specific
embodiments thereof, it will be understood that it is capable of further
modifications and this application is intended to cover any variations, uses, or
adaptations of the invention following, in general, the principles of the invention
and including such departures from the present disclosure as come within known
20 or customary practice within the art to which the invention pertains and as may be
applied to the essential features hereinbefore set forth.

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What Is Claimed Is:

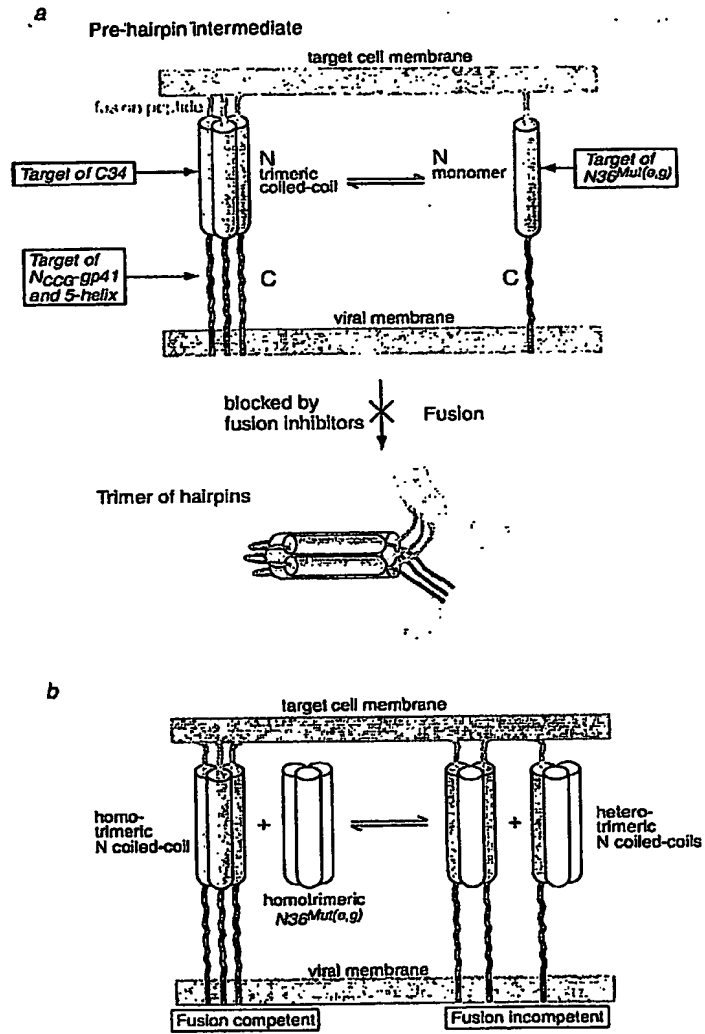
1. A gp41-targeted HIV fusion inhibitor.
2. A pharmaceutical composition comprising a gp41-targeted HIV fusion inhibitor.
- 5 3. A method of treating HIV infection that comprises providing to a recipient a therapeutically effective or a prophylactically effective amount of a pharmaceutical composition comprising a gp41-targeted HIV fusion inhibitor.

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Abstract of the Invention

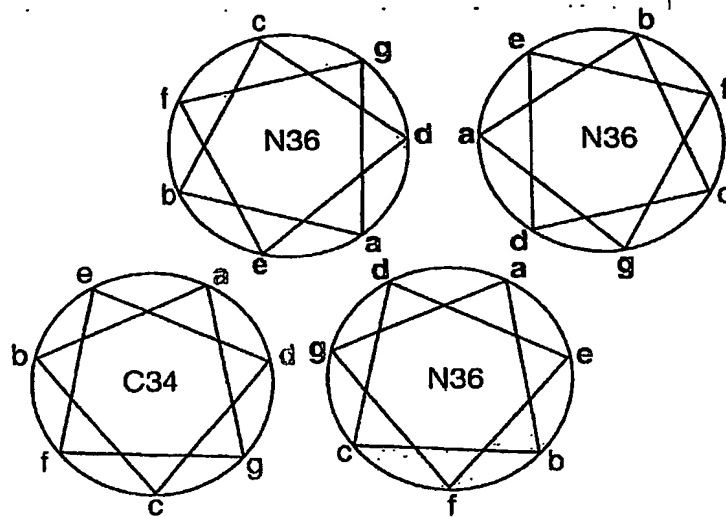
This invention relates to a novel peptide inhibitor of HIV fusion that
disrupts the internal trimeric coiled-coil of gp41, to a pharmaceutical composition
that comprise this inhibitor, and to methods of treating Immunodeficiency disease,
5 especially HIV, that employ such a pharmaceutical composition.

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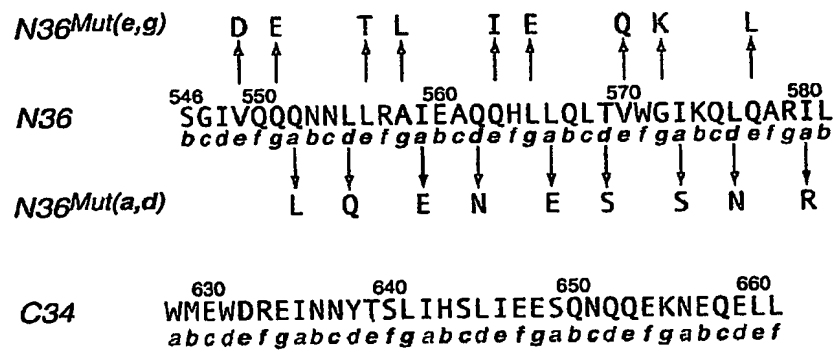


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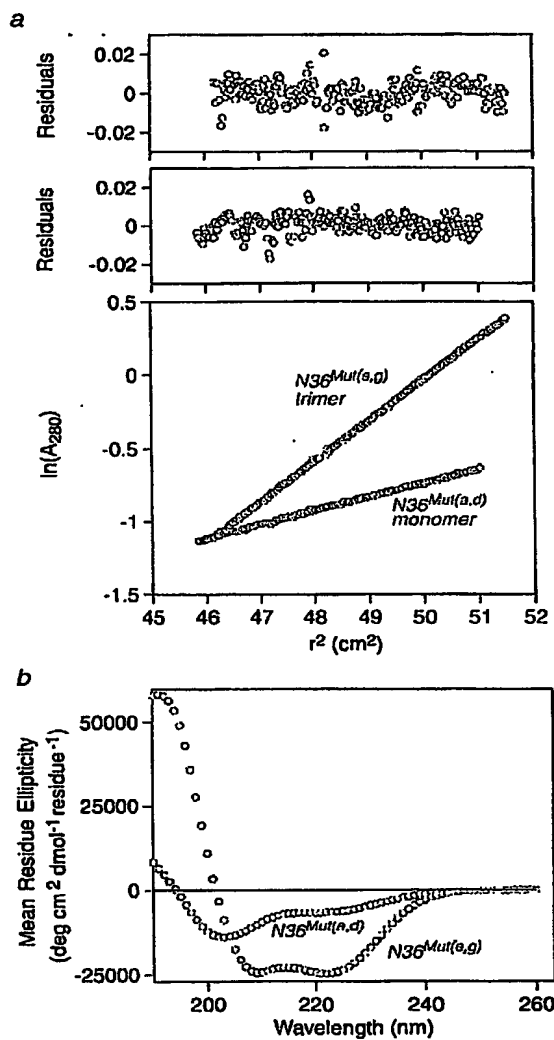
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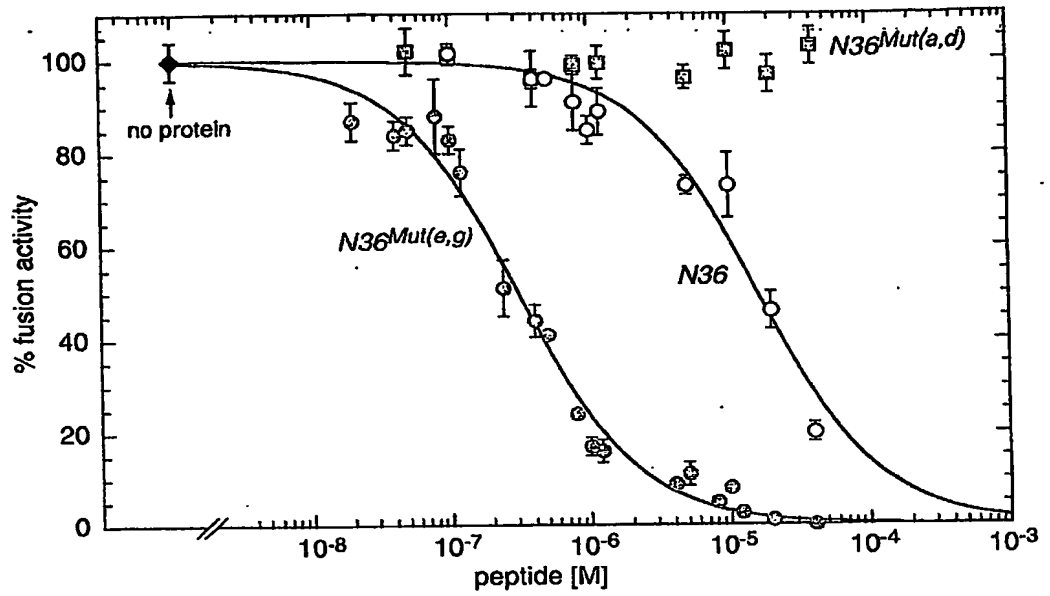
b



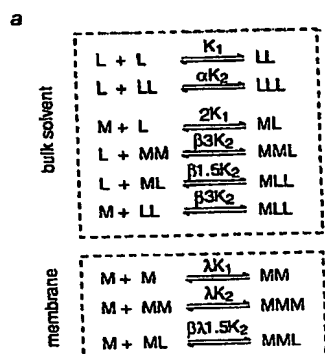
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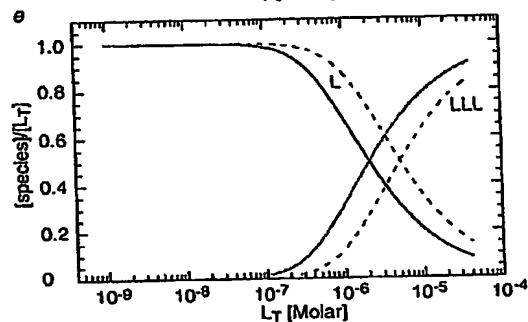
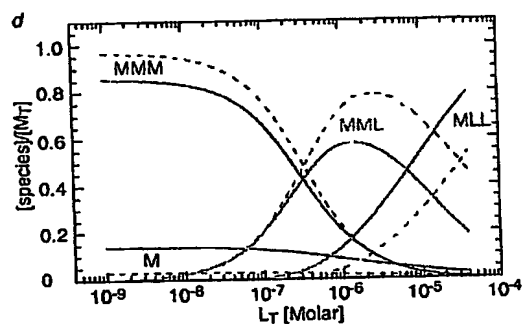
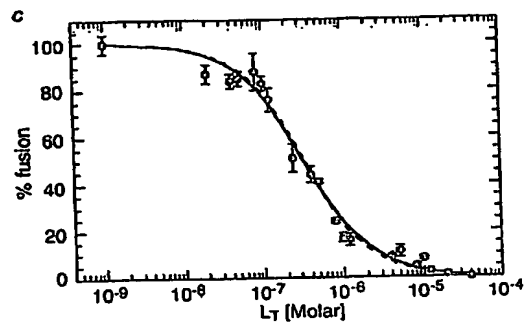
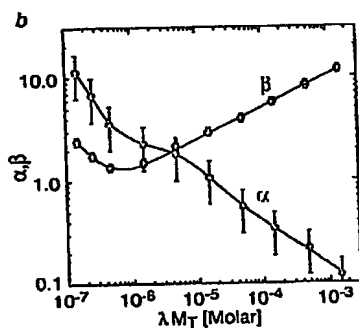


$$K_{\text{ref}}^{\text{trimer}} = K_1 K_2$$

$$K_{\text{homo,L}}^{\text{trimer}} = \alpha K_{\text{ref}}^{\text{trimer}}$$

$$K_{\text{hetero,ML}}^{\text{trimer}} = \beta 3 K_{\text{ref}}^{\text{trimer}}$$

$$K_{\text{homo,M}}^{\text{trimer,app}} = \lambda K_{\text{ref}}^{\text{trimer}}$$



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